Exhibit K

/REVIEW

Mammary Gland Expression of Transgenes and the Potential for Altering the Properties of Milk

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Transgenic animals are a useful *in vivo* experimental model for assessing the ability and impact of foreign gene expression in a biological system. Transgenic mice are most commonly used, while transgenic sheep, goats, pigs and cows have also been developed for specific, "applied" purposes. Most of the work directed at targeting expression of transgenes to the mammary gland of an animal, by using a milk gene promoter, has been with the intent of either studying promoter function or recovering the desired protein from the milk. Transgenic technology can also be used to alter the functional and physical properties of milk resulting in novel manufacturing properties. The properties of milk have been altered by adding a new protein with the aim of improving the milk, not of recovering the protein for other uses.

Transgenic animals are becoming more common and useful tools because they give an *in vivo* look at the ability and impact of foreign gene expression in a biological system. Expression of the gene of interest is controlled by DNA promoter elements that direct where and when the gene product will be expressed in the animal. Expression of a transgene in the mammary gland of an animal requires the use of a promoter and regulatory regions of a milk protein gene, i.e., sequences that direct gene expression only in the mammary gland and only during lactation.

Most of the work done with targeting transgene expression to the mammary gland of an animal has been concerned with either studying promoter function and identifying the important and necessary regions of DNA required for expression[1,2], or at producing biologically important and active proteins (such as pharmaceuticals) in the milk of a transgenic animal with the intent of recovering the protein of interest from the milk[2,3]. It is now also possible to use transgenic animals to alter the properties and composition of the milk itself by genetically adding a new, or altered, protein for its own effect rather than for recovery of the protein for other uses. More specifically, we are concerned with the addition of human lysozyme or a modified bovine κ-casein to bovine milk in order to affect the functional and physical properties of the milk protein system and thereby alter the manufacturing applications of milk.

Milk Genes and Promoters

Milk consists of six major proteins that are specific to the milk of most mammals. The proteins are grouped into two classes, the caseins, consisting of α_{s1}, α_{s2}, β and κ-casein, and the main whey proteins, α-lactalbumin and β-lactoglobulin. All milk protein genes are encoded by polymorphic, codominant allelic autosomal genes[4]. The milk protein gene promoters have been isolated and characterized (Fig. 1). The six major bovine milk protein genes are located on three separate chromosomes in the bovine genome[5], with the four casein genes located on chromosome 6 within 200 kb of each other in the order α_{s1}, β, α_{s2}, κ. This might suggest some common control or promoter element is used for the expression of the casein genes. The major whey protein genes were found to be located on separate chromosomes suggesting that each has its own distinct promoter. α-Lactalbumin is located on chromosome 3 and β-lactoglobulin on chromosome 16.

Transgenes Expressed in the Mammary Gland

A majority of the milk gene promoters have been used to generate transgenic animals that produce foreign proteins in the mammary gland (Table 1). Overall, it can be seen that the sheep β-lactoglobulin, goat β-casein and bovine α_{s1}-casein promoters are the most efficient at supporting good levels of heterologous protein expression in the mammary gland of transgenic animals[46,53,58]. Better expression may occur if genomic DNA sequences, rather than the cDNA of the desired protein, are used[62,63]. The incorporation of untranslated exons and introns may contribute to increased expression of the transgene[62]. The α-lactalbumin promoter is not yet as well characterized as the others, and the rat, rabbit and bovine β-casein promoters seem to work by promoting variable expression only at low levels in transgenic mice[50–52,59]. This suggests there may be an unidentified element(s) present that yields efficient expression in the α_{s1} and β-lactoglobulin promoters, but is missing in the regions of the β-casein 5′ DNA that have been used.

Milk Gene Products and Functions

The average composition of bovine milk is 86% water, 5% lactose, 4.1% fat, 3.6% protein and 0.7% minerals with a pH of 6.6–6.7. Milk composition remains relatively constant with the exception of fat content, which varies depending upon the breed of cow, feed type and stage of lactation[64]. All the components of milk are secreted by the mammary gland during lactation, with a typical cow yielding 10,000 pounds of milk over a 305-day lactation period.

There are six major gene products secreted by the mammary gland: α_{s1}, α_{s2}, β and κ-casein, β-lactoglobulin and α-lactalbumin which account for the 3.6% or 30–35 g/l of protein present in milk. The caseins contribute the majority of the protein, 80% or 24–28 g/l, while the whey proteins, β-lactoglobulin and α-lactalbumin, account for 20% of the total protein or 5–7 g/l. The caseins and whey proteins are designated as such since they can be separated into two distinct fractions based on the properties of each individual protein and how they interact with each other to form milk.

A new Web site, the Mammary Gland Biology Database, has been established by the Mammary Gland Information Core (MAGIC) and the Developmental Biology Section at the National Institutes of Health. The address of the Web site is (http://scrc.dcrt.nih.gov/~mammary).

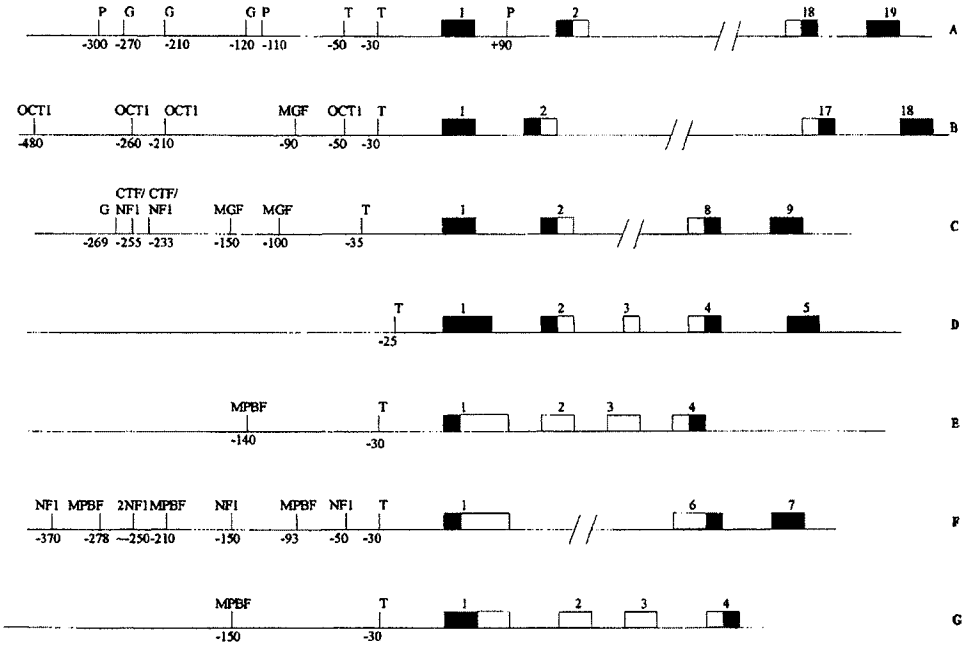

FIGURE 1. Characteristics of the milk protein genes. Schematic representation of the milk protein genes and promoters. The number of the individual exon is given over each box. Not all exons are shown. (A) Bovine α_{s1}-casein[5,7]. The gene is a total 17,508 bp long. The splicing of exons 9 and 10 creates a phosphorylation site, while there are also progesterone sites in introns, suggesting a regulatory role for introns. Information is also known for the rabbit gene[8]. (B) Bovine α_{s2}-casein[9]. The gene is 18,483 bp and is organized like bovine α_{s1}-casein, but sequence wise is more closely related to β-casein genes. (C) Bovine β-casein[10,11]. The gene is 8.6 kb long with a similar organization to the α-casein genes, with the splicing of exon 4 to 5 creating a phosphorylation site. The goat gene is 95% similar to the bovine gene and has a SV40 enhancer and mammary gland regulatory element located at −102 and −150 respectively[12]. Information is known for the rat[13], mouse[14], rabbit[15] and human[16] genes. (D) Bovine κ-casein[17]. The gene is 13 kb long and its structure is not related to the other casein genes. The length and amino acid composition of the signal sequence differ and the 3′ nontranslated region is shorter. There are no hormone binding sites in the 5′ nontranslated region. Information is known for the mouse and rat genes[18]. (E) Human α-lactalbumin[19]. The gene is 12 kb long and contains *Alu* elements at −656 and in the first exon, and shows the presence of a casein common regulatory element in a non-casein gene. Information is known for the rat[20], mouse[21], bovine[22], goat[23] and guinea pig[24] genes. (F) Sheep β-lactoglobulin[25,26]. The gene is 4.2 kb and essential expression elements are located within the −406 to −1 region. Information is also known for the bovine gene[27]. (G) Rat WAP[28]. Whey acidic protein is a cysteine-rich principal protein of rodent milk, but is not found in livestock or human milk. The gene is 2.8 kb long and has very few similarities to other milk protein genes. Information is known for the mouse[29] and rabbit[30] genes. Nontranslated exon (■); Translated exon (□); T = TATA box; P = Progesterone binding site; G = Glucocorticoid binding site; OCT1 = OCT1 consensus site; NF1 = NF1 binding site; CTF/NF1 = binding site; MGF = Mammary gland factor; MPBF = Milk protein binding factor.

Caseins. The caseins are single polypeptide chains that have very little secondary structure, high average hydrophobicity and a net negative charge. Caseins contain many essential amino acids but are low in cystine and high in proline content, which acts as a secondary structure breaker[65]. The caseins are post-translationally phosphorylated in the Golgi at accessible serine residues in the sequence Ser/Thr-X-Glu/Ser-PO_4, where X is any amino acid residue[66]. The phosphorylation is essential as it allows for the calcium-binding ability and other functions of the caseins.

The caseins are present in milk in the form of micelles, or a colloidal suspension of proteins. The micelles are on the order of 20–600 nm in size and are composed of approximately 93% casein and 7% of a combination of calcium (Ca), phosphate (PO_4) and colloidal calcium phosphate ($CaPO_4$)[65]. The caseins are present in a ratio of α_{s1}:α_{s2}:β:κ at 3:1:3:1. Hydrophobic interactions and hydrogen bonding are important in keeping micelles together[67]. Because of their unordered and highly hydrophobic nature, the caseins can aggregate with themselves and the other caseins by hydrophobic interactions, hydrogen bonding, electrostatic attractions (Ca to Ser-PO_4) or repulsions (Ser-PO_4 to Ser-PO_4) and disulfide bonding. α_{s1} and β-casein tend to self associate while α_{s1} and κ-casein form aggregates with each other. α_{s1}, α_{s2} and β-casein form the core of the micelle while the amphiphilic κ-casein lies on the surface.

Micelles function to sequester and transport calcium in a usable form to the newborn for bone development. The structure of the micelle can be disrupted by acid or the enzyme rennin. Acid, at pH 4.6, acts to neutralize the negative charges of the phosphate and promote association leading to isoelectric coagulation of the caseins. The acid pH of the stomach causes casein precipitation, which makes the milk protein more digestible. Rennin is a combination of chymosin and pepsin which specifically cleaves κ-casein at the Phe105-Met106 peptide bond. Once cleaved, para κ-casein, or the hydrophobic N-terminal of the protein, stays associated with the micelle and the hydrophilic C-terminal is released from the micelle. Cleavage of κ-casein causes destabilization of the micelle structure. The hydrophilic portion of κ-casein "sticks off" the micelle surface and causes repulsion among the other caseins so they cannot aggregate at all possible sites. With cleavage, the repulsive action is no longer associated with the micelle, more contact among the caseins can occur and calcium can have access to all the phosphorylated sites on the caseins. With calcium binding at all the sites, precipitation of the caseins occurs and separates the milk into two distinct fractions, the nonsoluble caseins and the soluble whey proteins.

Whey proteins. The whey proteins are usually compact and globular with a relatively uniform distribution of polar, nonpolar and charged residues[65]. This is opposed to the clustering of similar residues that characterize the caseins. The

whey proteins tend to be high in cystine content, whose disulfide bonds can contribute to flavor. Whey proteins are more hydrophilic than the caseins as their hydrophobic residues tend to be buried rather than exposed and bind more water. The whey proteins are so named due to the fact that they stay in solution after the caseins are precipitated by acid or rennet.

Candidates for Altering the Properties of Milk

Lysozyme. Lysozyme has been shown to have antimicrobial activity[68] and is positively charged at physiological pH[69]. If human lysozyme were present in bovine milk at a significant level, two main effects could be considered. First, because of its antimicrobial activity, lysozyme may help to reduce the overall level of bacteria in milk and decrease disease in the udder. Second, because of its positive charge, lysozyme may be able to interact with the negatively charged caseins to produce a milk with novel functional and physical properties.

Although their biological function is not exactly known, it is thought that lysozymes serve as defense mechanisms against bacterial infection[70] or for digestion of intestinal bacteria[71]. Lysozymes are ubiquitous enzymes found in avian egg whites and mammalian secretions such as tears, saliva and milk, as well as others[69]. Lysozymes specifically hydrolyze the glycosidic linkages between the C-1 of N-acetylmuramic acid and the C-4 of N-acetylglucosamine, which make up the peptidoglycan component of bacterial cell walls[68]. Cleavage of the protective peptidoglycan layer causes leakage of the cells interior components and results in cell lysis. Lysozyme tends to be more effective against gram positive bacteria[72] as the outer membrane, found in gram negative bacteria, is not present and the enzyme has free access to the peptidoglycan. Lysozyme can be inhibited by surface active reagents such as SDS, alcohols and fatty acids[73].

Antimicrobial effect. Lysozyme is naturally present in human milk at levels 3000 times greater than it is in bovine milk[74]. Human milk contains 400 μg/ml lysozyme while bovine milk on the average contains only 0.130 μg/ml, goat milk 0.250 μg/ml, sheep milk 0.10 μg/ml and sow and rodent milk contains little if any lysozyme[74]. Several microorganisms were found to be susceptible to human and bovine milk lysozymes and it is suggested that lysozyme is part of the inherent antimicrobial activity of milk[75]. Lysozyme is considered to be part of passive immunity and the natural defense against bacteria, virus, parasites and fungi in human milk[76].

Results of several studies show lysozyme's lytic effect on food microorganisms or the effect of milk on these organisms[77–83]. Lysozyme has been shown to inhibit growth of *Listeria monocytogenes* in several foods[77] and was effective against bacteria causing food borne disease and spoilage at levels of 10–200 mg/l[78]. At 100 mg/l milk, lysozyme inhibited growth of non-acid-forming bacteria

Lysozyme isolated from rainbow trout kidney was active against several bacterial strains that cause mastitis, including *Staphylococcus aureus, E. coli* and two strains of *Streptococcus*[80]. The bacteria were more resistant to the lysozyme when grown in milk than when grown in medium alone, probably due to the formation of a protective capsule induced by the milk. However, if lysozyme was present in the udder, it might be effective at the start of infection, before great numbers of bacteria accumulate.

Human milk has been shown to be antibacterial[81], being more effective at stopping the growth of *Bordetella pertussis* than was bovine milk[81]. Together, lysozyme and lactoferrin were inhibitory at concentrations normally occurring in human milk[82] and the antibacterial activity of these two proteins are considered to be the most important factors in the nonspecific immunity of milk[83]. It may be concluded that lysozyme can be active as an additive to foods to act to lower the levels of the bacteria or inhibit their initial growth. The use of lysozyme as a food preservative or additive has also been

TABLE 1. Mammary gland-specific transgenic animals.

Source of Promoter	Amount of Promoter	To Express	Animal	Protein Levels	Reference
WAP-rat	Entire gene	Rat WAP	Mice	27% of endogenous	31
WAP-mouse	2.5 kb 5′	Ha-*ras* oncogene	Mice	Produced tumors	32
WAP-mouse	2.6 kb 5′, Sv40 PolyA	cDNA t-PA	Mice	300 ng/ml	33
WAP-mouse	2.6 kb 5′, Sv40 PolyA	cDNA t-PA	Goats	3 μg/ml	34
WAP-mouse	Entire gene	Mouse WAP	Pigs	1 g/l	35
WAP-mouse	2.3 kb 5′, 1.6 kb 3′	cDNA human SOD	Mice	0.7 mg/ml	36
WAP-mouse	2.6 kb 5′, 1.6 kb 3′	cDNA human proteinC	Mice	3 μg/ml	37
WAP-mouse	2.6 kb 5′, 1.6 kb 3′	cDNA human proteinC	Pigs	0.001–1 mg/ml	38
WAP-mouse	4.2 kb 5′	gDNA human proteinC	Mice	0.1–0.7 mg/ml	39
WAP-rabbit	17.6 kb 5′	gDNA human α1-antitrypsin	Mice	10 mg/ml	40
WAP-rabbit	6.3 kb	gDNA human growth hormone	Mice	4–22 mg/ml	41
α-lactalbumin-guinea pig	1195 bp 5′, 395 bp 3′	Guinea pig α-lactalbumin	Mice	NQ	42
α-lactalbumin-bovine	Entire gene	Bovine α-lactalbumin	Mice	1.5 mg/ml	43
α-lactalbumin-bovine	750 bp 5′, 336 bp 3′	cDNA bovine α-lactalbumin	Mice	0.0025–0.45 mg/ml	44
α-lactalbumin-bovine	477 bp 5′, 336 bp 3′	cDNA bovine α-lactalbumin	Mice	0.1 mg/ml	45
α-lactalbumin-goat	Entire gene	Goat α-lactalbumin	Mice	1.2–3.7 mg/ml	45
β-lactalbumin-sheep	4 kb 5′, 1.9 or 7.3 kb 3′	Sheep β-lactalbumin	Mice	23 mg/ml	46
β-lactoglobulin-sheep	4 kb 5′	Human α1-antitrypsin	Mice	0.4–7.3 mg/ml	47
β-Lactoglobulin-sheep	4 kb 5′	Human α1-antitrypsin	Sheep	35 g/l	48
β-Lactoglobulin-sheep	3, 5.5 or 10.8 kb 5′, 8 kb 3′	Sheep β-lactoglobulin	Mice	1–8.5 mg/ml	49
β-Lactoglobulin-sheep	3 kb 5′	cDNA human serum albumin	Mice	No expression	49
β-Lactoglobulin-sheep	3 kb 5′	gDNA human serum albumin	Mice	2.5 mg/ml	49
β-Lactoglobulin-sheep	1.8 kb 5′, 4.6 kb WAP 3′	cDNA human SOD	Mice	10 ng/ml	36
β-Casein-rat	3.5 kb 5′, 3 kb 3′	Rat β-Casein	Mice	0.01–1% endogenous (mRNA)	50
β-Casein-rat	2.3 kb 5′, 5.5 kb 5′	Bacterial CAT	Mice	Variable	51
β-Casein-rabbit	2 kb 5′	Human interkeukin-2	Rabbits	430 ng/ml	52
β-Casein-goat	3 kb 5′, 6 kb 3′	Goat β-casein	Mice	12–24 mg/ml	53
β-Casein-goat	4.2 kb 5′, 5.3 kb 3′	Goat β-casein	Mice	1 mg/ml	12
β-Casein-goat	4 kb 5′, 4.5 kb 3′	cDNA CFTR	Mice	NQ	54
β-Casein-goat	6.2 kb 5′, 7.1 kb 3′	cDNA bovine κ-casein	Mice	0.9–1.5 μg/μl	55
α_{s1}-Casein-bovine	1.35 kb 5′, 1.5 kb 3′	cDNA bovine α_{s1}-casein	Mice	0.1% endogenous (mRNA)	56
α_{s1}-Casein-bovine	1.35 kb 5′, Sv40 3′	Bacterial CAT	Mice	3 ng/mg	56
α_{s1}-Casein-bovine	2.9 kb 5′, 3.5 kb 3′	cDNA human IGF-1	Rabbits	1 g/l	57
α_{s1}-Casein-bovine	21 kb 5′, 2 kb 3′	gDNA human urokinase	Mice	1–2 mg/ml	58
α_{s1}-Casein-bovine	21 kb 5′, 2 kb 3′	cDNA human lysozyme	Mice	0.25–0.71 μg/μl	59
α_{s1}-Casein-bovine	6.2 or 14.2 kb 5′, 6.5 kb 3′	cDNA human lactoferrin	Mice	0.1–36 μg/ml	60
α_{s1}-Casein-bovine	15 kb 5′, 6 kb 3′	cDNA human lactoferrin	Cow	ND	61

noted. Lysozyme is highly water soluble, is tolerant to heat in acidic conditions, can survive and retain activity at pasteurization temperatures[84] and can be broken down by the human stomach[85]. Animal studies have shown that lysozyme at levels of 5 mg/g body weight can be taken with no toxic effects[86].

Alteration of physical and functional properties. Human lysozyme could also alter the physical and, ultimately, the functional properties of the bovine milk protein system. Lysozyme has a net positive charge at physiological pH[69], which may allow it to interact with the negatively charged caseins and effect the charge repulsion favoring interactions between the various milk proteins. *In vitro* experiments indicate this could potentially affect dairy processes such as rennet clotting time, isoelectric precipitation and cheese yield[87–91].

Polycations such as lysozyme, salmine and calcium have been shown to cause precipitation and reduce the rennet clotting time[87]. The rennet clotting time, or time for curd formation, was reduced by 80% with the addition of 0.695 mM lysozyme. It was also found that almost all of the added lysozyme was absorbed by the micelles. Negatively charged substances such as SDS[88] and negatively charged proteins such as α-lactalbumin and β-lactoglobulin[89] were found to increase the rennet clotting time. Electrostatic interactions were further seen as coagulation of milk proteins was observed using cationic polyelectrolytes[90]. It has also been demonstrated that lysozyme could be beneficial for rennet clotting time, cheese yield and syneresis[91]. At 500–2000 ppm, lysozyme decreased rennet clotting time by one to four minutes over controls, and at 20 ppm was found to bind to caseins increasing cheese yield. Lysozyme demonstrated these same effects when actual cheese making was carried out in a vat[91], suggesting that lysozyme could in fact be used as a technological aid in cheese processing.

κ-Casein. The addition of more bovine κ-casein to the milk protein system could also affect the physical properties of the milk since κ-casein is directly involved with micelle formation[92], structure[92,93], size[65], and therefore, milk function. An increase in κ-casein could increase the thermal stability of casein aggregates[94–97] and act to decrease micelle size[94]. A smaller micelle diameter would lead to a larger available surface area, which would result in a more consistent and firmer curd as well as an increase in cheese yield[98,99]. These modified properties of milk could be of great benefit and interest to the dairy industry.

Lactoferrin. The presence of lactoferrin could also be considered for altering the microbial and nutritional properties of bovine milk. Lactoferrin is the major iron binding protein in milk. Lactoferrin has two high affinity iron binding sites[100]. Lactoferrin inhibits the growth of bacteria in the mammary gland and the intestine of infants[101], and mediates iron transport and absorption in the newborn[100]. Lactoferrin can also act as an antioxidant, inhibiting the formation of toxic oxygen radicals[102]. Lactoferrin is present in human milk at levels of 1.7 mg/ml while cow's milk contains only 0.02–0.2 mg/ml[103].

Lactoferrin inhibits bacterial growth by either binding to the outer membrane of gram negative bacteria[104] or by lowering the availability of iron for those bacteria which need iron for growth[105]. The bacteriostatic or bacteriocidal effects of lactoferrin have been noted as important in the natural defense system in mammalian milk[106]. The presence of human lactoferrin in bovine milk could act to decrease mastitis in the cow and decrease the overall level of bacteria in the milk. Lactoferrin may also act in the newborn to decrease or prevent enteric infections.

Lactoferrin is responsible for the high bioavailability of milk iron[100] and it is thought that supplementing infant formulas with lactoferrin may help prevent iron deficiency in infants fed cow milk formula[107]. The presence of human lactoferrin in milk could lead to a better source of iron for the newborn by increasing the dietary source and the intestinal absorption of iron.

Human lactoferrin, like human lysozyme, may also be able to alter the processing properties of milk based on charge interactions between it and the milk proteins. Human lactoferrin is slightly basic (pI=8.7), while human lyoszyme is strongly basic (pI=11). Lactoferrin itself has been shown to to able to associate with proteins including caseins, α-lactalbumin[108]. The presence of human lactoferrin could enhance association with the caseins which could lead to an increase in cheese yield, or gel strength, among other important processing properties as discussed above.

Conclusions

In transgenic mice expressing human lysozyme in their milk[59], the rennet clotting time of milk was decreased by 35%, gel strength was significantly higher than in control mice, while the particle size of micelles remained the same[109]. Results from mice expressing the bovine κ-casein gene[55] also showed significantly different gel strength as well as particle size, but no difference in rennet clotting time when compared to control mice[110]. Transgenic mice[60] and cattle[61] have produced human lactoferrin, but no studies on the functional properties of the resulting milk have been reported.

These results in mice suggest that human lysozyme and κ-casein are good candidates for beneficially altering properties of milk. Transgenic technology can be used with success for applied purposes as well as for studying gene function and for the production and recovery of novel proteins in the milk of transgenic livestock.

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References

1. Mercier, J. C. and Vilotte, J. L. 1993. Structure and function of milk protein genes. J. Dairy Sci. **76**:3079–3098.
2. Bawden, W. S., Passey, R. J. and Mackinlay, A. G. 1994. The genes encoding the major milk specific proteins and their use in transgenic studies and protein engineering. Biotech. Gen. Eng. Rev. **12**:89–137.
3. Wilmut, I., Archibald, A. L., McClenaghan, M., Simons, J. P., Whitelaw, C. B. A. and Clark, A. J. 1991. Production of pharmaceutical proteins in milk. Experientia **47**:905–912.
4. Fennema, O. R. 1985. *In*: Food Chemistry, 2nd Edition, Fennema, O. R. (Ed.). M. Dekker, New York.
5. Threadgill, D. W. and Womack, J. E. 1990. Genomic analysis of the major bovine milk protein genes. Nucleic Acids Res. **23**:6935–6942.
6. Lee, L.-Y. and Rosen, J. M. 1988. A transfected α-casein minigene bypasses post-transcriptional control by hormones, but retains cell-substratum regulation in mammary epithelial cells. Mol. Endocrinol. **2**:431–443.
7. Koczan, D., Hobom, G. and Seyfert, H.-M. 1991. Genomic organization of the bovine α_{s1}-casein gene. Nucleic Acids Res. **19**:5591–5596.
8. Jolivet, G., Devinoy, E., Fontaine, M. L. and Houdebine, L. M. 1992. Structure of the gene encoding rabbit α_{s1}-casein. Gene **113**:257–262.
9. Groenen, M. A. M., Dijkhof, R. J. M., Verstege, A. J. M. and van der Poel, J. J. 1993. The complete sequence of the gene encoding bovine α_{s2}-casein. Gene **123**:187–193.
10. Bonsing, J., Ring, J. M., Stewart, A. F. and Mackinlay, A. G. 1988. Complete nucleotide sequence of the bovine β-casein gene. Aust. J. Biol. Sci. **41**:527–537.
11. Gorodetsky, S. I., Tkach, T. M. and Kapelinskaya, T. V. 1988. Isolation and characterization of the *Bos taurus* β-casein gene. Gene **66**:87–96.
12. Roberts, B., DiTullio, P., Vitale, J., Hehir, K. and Gordon, K. 1992. Cloning of the goat β-casein encoding gene and expression in transgenic mice. Gene **121**:255–262.
13. Jones, W. K., Yu-Lee, L.-Y., Clift, S. M., Brown, T. L. and Rosen, J. M. 1985. The rat casein multigene family. Fine structure and evolution of the β-casein gene. J. Biol. Chem. **260**:7042–7050.
14. Yoshimura, M. and Oka, T. 1989. Isolation and structural analysis of the mouse β-casein gene. Gene **78**:267–275.
15. Thepot, D., Devinoy, E., Fontaine, M. L. and Houdebine, L. M. 1991. Structure of the gene encoding rabbit β-casein. Gene **97**:301–306.
16. Hansson, L., Edlund, A., Johansson, T., Hernell, O., Stromquist, M., Lindquist, S., Lonnerdal, B. and Bergstrom, S. 1994. Structure of the human β-casein encoding gene. Gene **13**:193–199.
17. Alexander, J., Stewart, A. F., Mackinlay, A. G., Kapelinskaya, T. V., Tkach, M. and Gorodetsky, S. I. 1988. Isolation and characterization of the bovine κ-casein gene. Biochem. J. **178**:395–401.
18. Thompson, M. D., Dave, J. R. and Nakhasi, H. L. 1985. Molecular cloning of

mouse mammary gland κ-casein: comparison with rat κ-casein and rat and human γ-fibrinogen. DNA **4**:263–271.
19. Hall, L., Emery, D. C., Davies, M. S., Parker, D. and Craig, R. K. 1987. Organization and sequence of the human α-lactalbumin gene. Biochem. J. **242**:735–742.
20. Quasba, P. K. and Safaya, S. K. 1984. Similarity of the nucleotide sequences of the rat α-lactalbumin and chicken lysozyme genes. Nature **308**:377–380.
21. Vilotte, J. L. and Soulier, S. 1992. Isolation and characterization of the mouse α-lactalbumin-encoding gene: interspecies comparison, tissue- and stage-specific expression. Gene **119**:287–292.
22. Vilotte, J. L., Soulier, S., Mercier, J. C., Gaye, P., Hue-Delahaie, D. and Furet, J. P. 1987. Complete nucleotide sequence of bovine α-lactalbumin gene. Comparison with its rat counterpart. Biochimie **69**:609–620.
23. Vilotte, J. L., Soulier, S., Printz, C. and Mercier, J. C. 1991. Sequence of the goat α-lactalbumin-encoding gene: comparison with the bovine gene and evidence of related sequences in the goat genome. Gene **98**:271–276.
24. Laird, J. E., Lack, L., Hall, L., Boulton, A., Parker, D. and Craig, R. K. 1988. Structure and expression of the guinea pig α-lactalbumin gene. Biochem. J. **254**:85–94.
25. Harris, S., Ali, S., Anderson, S., Archibald, A. L. and Clark, A. J. 1988. Complete nucleotide sequence of the ovine β-lactoglobulin gene. Nucleic Acids Res. **16**:10379–10380.
26. Watson, C. J., Gordon, K. E., Robertson, M. and Clark, A. J. 1991. Interaction of DNA binding proteins with a milk protein gene promoter *in vitro*: identification of a mammary gland-specific factor. Nucleic Acids Res. **19**:6603–6610.
27. Alexander, L. J., Hayes, G., Bawden, W., Stewart, A. F. and Mackinlay, A. G. 1993. Complete nucleotide sequence of the bovine β-lactoglobulin gene. Animal Biotech. **4**:1–10.
28. Hennighausen, L. G. and Sippel, A. E. 1982. Characterization and cloning of the mRNAs specific for the lactating mouse mammary gland. Eur. J. Biochem. **125**:131–141.
29. Campbell, S. M. and Rosen, J. M. 1984. Comparison of the whey acidic protein genes of the rat and mouse. Nucleic Acids Res. **12**:8685–8696.
30. Thepot, D., Devinoy, E., Fontaine, M. L. and Houdebine, L. M. 1991. Complete sequence of the rabbit whey acidic protein gene. Nucleic Acids Res. **18**:3641.
31. Bayna, E. M. and Rosen, J. M. 1990. Tissue specific expression of the rat whey acidic protein gene in transgenic mice. Nucleic Acids Res. **18**:2977–2985.
32. Andres, A.-C., Schonenberger, C.-A., Groner, B., Hennighausen, L., LeMeur, M. and Gerlinger, P. 1987. Ha-ras oncogene expression directed by a milk protein gene promoter: Tissue specificity, hormonal regulation, and tumor induction in transgenic mice. Proc. Natl. Acad. Sci. USA **84**:1299–1303.
33. Gorden, K., Lee, E., Vitale, J. A., Smith, A. E., Westphal, H. and Hennighausen, L. 1987. Production of human tissue plasminogen activator in transgenic mouse milk. Bio/Technology **5**:1183–1187.
34. Ebert, K. M., Selgrath, J. P., DiTullio, P., Denman, J., Smith, T. E., Memon, M. A., Schindler, J. E., Monastersky, G. M., Vitale, J. A. and Gorden, K. 1991. Transgenic production of a variant of human tissue type plasminogen activator in goat milk: Generation of transgenic goats and analysis of expression. Bio/Technology **9**:835–838.
35. Wall, R. J., Pursel, V. G., Shamay, A., McKnight, R. A., Pittius, C. W. and Hennighausen, L. 1991. High level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. Proc. Natl. Acad. Sci. USA **88**:1696–1700.
36. Hansson, L., Edlund, M., Edlund, A., Johansson, T., Marklund, S. L., Fromm, S., Stromqvist, M. and Tornell, J. 1994. Expression and characterization of biologically active human extracellular superoxide dismutase in milk of transgenic mice. J. Biol. Chem. **69**:5358–5363.
37. Velander, W. H., Page, R. L., Morcol, T., Russell, C. G., Canseco, R., Young, J. M., Drohan, W. N., Gwazdauskas, F. C., Wilkins, T. D. and Johnson, J. L. 1992. Production of biologically active protein C in the milk of transgenic mice. Ann. N. Y. Acad. Sci. **665**:391–403.
38. Velander, W. H., Johnson, J. L., Page, R. L., Russell, C. G., Subramanian, A., Wilkins, T. D., Gwazdauskas, F. C., Pittius, C. and Drohan, W. N. 1992. High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. Proc. Natl. Acad. Sci. USA **89**:12003–12007.
39. Drohan, W. N., Zhang, D., Paleyanda, R. K., Chang, R., Wroble, M., Velander, W. and Lubon, H. 1994. Inefficient processing of human protein C in the mouse mammary gland. Transgenic Res. **3**:355–364.
40. Bischoff, R., Degryse, E., Perraud, F., Dalemans, W., Ali-Hadji, D., Thepot, D., Devinoy, E., Houdebine, L. M. and Pavirani, A. 1992. A 17.6 kbp region located upstream of the rabbit WAP gene directs high level expression of a functional human protein variant in transgenic mouse milk. FEBS **305**:265–268.
41. Devinoy, E., Thepot, D., Stinnakre, M.-G., Fontaine, M.-L., Grabowski, H., Puissant, C., Pavirani, A. and Houdebine, L.-M. 1994. High level production of human growth hormone in the milk of transgenic mice: the upstream region of the rabbit whey acidic protein (WAP) gene targets transgene expression to the mammary gland. Transgenic Res. **3**:79–89.
42. Maschio, A., Brickell, P. M., Kioussis, D., Mellor, A. L., Katz, D. and Graig, R. K. 1991. Transgenic mice carrying the guinea-pig α-lactalbumin gene transcribe milk protein genes in their sebaceous glands during lactation. Biochem. J. **275**:459–467.
43. Bleck, G. T. and Bremel, R. D. 1993. Variation in expression of a bovine α-lactalbumin transgene in milk of transgenic mice. J. Dairy Sci. **77**:1897–1904.
44. Vilotte, J. L., Soulier, S., Stinnakre, M. G., Massoud, M. and Mercier, J. C. 1989. Efficient tissue-specific expression of bovine α-actalbumin in transgenic mice. Eur. J. Biochem. **186**:43–48.
45. Soulier, S., Vilotte, J. L., Stinnakre, M. G. and Mercier, J. C. 1992. Expression analysis ruminant α-lactalbumin in transgenic mice: developmental regulation and general location of important *cis*-egulatory elements. FEBS **297**:13–18.
46. Simons, J. P., McClenaghan, M. and Clark, A. J. 1987. Alteration of the quality of milk by expression of sheep β-lactoglobulin in transgenic mice. Nature **328**:530–532.
47. Archibald, A. L., McClenaghan, M., Hornsey, V., Simons, J. P. and Clark, A. J. 1990. High level expression of biologically active human α_1-antitrypsin in the milk of transgenic mice. Proc. Natl. Acad. Sci. USA **87**:5178–5182.
48. Wright, G., Carver, A., Cottom, D., Reeves, D., Scott, A., Simons, P., Wilmut, I., Garner, I. and Colman, A. 1991. High level expression of active human α_1-antitrypsin in the milk of transgenic sheep. Bio/Technology **9**:830–834.
49. Shani, M., Barash, I., Nathan, M., Ricca, G., Searfoss, G. H., Dekel, I., Faerman, A., Givol, D. and Hurwitz, D. R. 1992. Expression of human serum albumin in the milk of transgenic mice. Transgenic Res. **1**:195–208.
50. Lee, K.-F., DeMayo, F. J., Atiee, S. H. and Rosen, J. M. 1988. Tissue specific expression of the rat β-casein gene in transgenic mice. Nucleic Acids Res. **16**:1027–1041.
51. Lee, K.-F., Atiee, S. H. and Rosen, J. M. 1989. Differential regulation of rat β-casein-chloramphenicol acetyltransferase fusion gene expression in transgenic mice. Mol. Cell Biol. **9**:560–565.
52. Buhler, T. A., Bruyere, T., Went, D. F., Stranzinger, G. and Burki, K. 1990. Rabbit β-casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits. Bio/Technology **8**:140–143.
53. Persuy, M.-A., Stinnakre, M.-G., Printz, C., Mahe, M.-F. and Mercier, J. C. 1992. High expression of the caprine β-casein gene in transgenic mice. Eur. J. Biochem. **205**:887–893.
54. DiTullio, P., Cheng, S. H., Marshall, J., Gregory, R. J., Ebert, K. M., Meade, H. M. and Smith, A. E. 1992. Production of cystic fibrosis transmembrane conductance regulator in the milk of transgenic mice. Bio/Technology **10**:74–77.
55. Gutierrez, A., Meade, H. M., Jimenez-Flores, R., Anderson, G. B., Murray, J. D. and Medrano, J. F. 1995. Expression analysis of bovine κ-casein in the mammary gland of transgenic mice. Transgenic Res. *In press.*
56. Clarke, R. A., Sokol, D., Rigby, N., Ward, K., Murray, J. D. and Mackinlay, A. G. 1994. Mammary gland specific expression of bovine α_{s1}-casein derived transgenes in mice. Transgenics **1**:313–319.
57. Brem, G., Hartl, P., Besenfelder, U., Wolf, E., Zinovieva, N. and Pfaller, R. 1994. Expression of synthetic cDNA sequences encoding human insulin-like growth factor-I (IGF-1) in the mammary gland of transgenic rabbits. Gene **149**:351–355.
58. Meade, H., Gates, L., Lacy, E. and Lonberg, N. 1990. Bovine α_{s1}-casein gene sequences direct high level expression of active human urokinase in mouse milk. Bio/Technology **8**:443–446.
59. Maga, E. A., Anderson, G. B., Huang, M. C. and Murray, J. D. 1994. Expression of human lysozyme mRNA in the mammary gland of transgenic mice. Transgenic Res. **3**:36–42.
60. Platenburg, G. J., Kootwijk, E. P. A., Kooiman, P. M., Woloshuk, S. L., Nuijens, J. H., Krimpenfort, P. J. A., Pieper, F. R., de Boer, H. A. and Strijker, R. 1994. Expression of human lactoferrin in milk of transgenic mice. Transgenic Res. **3**:99–108.
61. Krimpenfort, P., Rademakers, A., Eyestone, W., van der Schans, A., van den Broek, S., Kooiman, P., Kootwijk, E., Platenburg, G., Pieper, F., Strijker, R. and de Boer, H. 1991. Generation of transgenic dairy cattle using *in vitro* embryo production. Bio/Technology **9**:844–847.
62. Whitelaw C. B. A., Archibald, A. L., Harris, S., McClenaghan, M., Simons, J. P. and Clark, A. J. 1991. Targeting expression to the mammary gland: intronic sequences can enhance the efficiency of gene expression in transgenic mice. Transgenic Res. **1**:3–13.
63. Hurwitz, D. R., Nathan, M., Barash, I., Ilan, N. and Shani, M. 1994. Specific combinations of human serum albumin introns direct high level expression of albumin in transfected COS cells and in the milk of transgenic mice. Transgenic Res. **3**:365–375.
64. Webb, B. H., Johnson, A. H. and Alford, J. A. 1974. *In*: Fundamentals in Dairy Chemistry, 2nd Edition, Webb, B. H., Johnson, A. H. and Alford, J. A. (Eds.). AVI Publishing Co. Inc., Westport, Connecticut.
65. Fox, P. F. 1982. Chemistry of milk protein. Vols. 1 and 2 *In*: Developments in Dairy Chemistry, Fox, P. F. (Ed.). Applied Sci. Pub., New York.
66. Mercier, J. C. 1981. Phosphorylation of caseins: evidence for an amino acid triplet code posttranslationally recognized by specific kinases. Biochimie **63**:1–17.
67. Holt, C. 1992. Structure and stability of bovine casein micelles. Adv. in Prot. Chem. **43**:63–157.
68. Phillips, D. C. 1966. The 3-dimensional structure of an enzyme molecule. Sci. Am. **215**:78–90.
69. Jolles, P. and Jolles, J. 1984. What's new in lysozyme research. Mol. and Cell. Biochem. **63**:165–189.
70. Fleming, A. 1922. On a remarkable bacteriolytic element found in tissues and secretions. Proc. Roy. Soc. London Ser. B **93**:306–317.
71. Dobson, D. E., Prager, E. M. and Wilson, A. C. 1984. Stomach lysozyme of ruminants, I. Distribution and catalytic properties. J. Biol. Chem. **259**:11607–11616.
72. Costerton, J. W., Ingram, J. M. and Chang, K. J. 1974. Structure and function of the cell envelope of bacteria. Bacteriol. Rev. **38**:87–110.
73. Smith, G. N. and Stoker, C. 1949. Inhibition of crystalline lysozyme. Arch. Biochem. **21**:383.
74. Chandan, R. C., Parry, R. M. and Shahani, K. M. 1968. Lysozyme, lipase, and ribonuclease in milk of various species. J. Dairy Sci. **51**:606–607.
75. Vakil, J. R., Chandan, R. C., Parry, R. M. and Shahani, K. M. 1970. Susceptibility of several microorganisms to milk lysozymes. J. Dairy Sci. **52**:1192–1197.
76. Chang, S. S. 1990. Antimicrobial proteins of maternal and cord sera and human milk in relation to maternal nutritional studies. Am. J. Clin. Nutr.

51:183–187.
77. Hughey, V. L., Wilger, P. A. and Johnson, E. A. 1989. Antibacterial activity of hen egg white lysozyme against *Listeria monocytogenes* ScottA in foods. Appl. Environ. Microbiol. **55**:631–638.
78. Hughey, V. L. and Johnson, E. A. 1987. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food borne disease. Appl. Environ. Microbiol. **53**:2165–2170.
79. Panfil-Kuncewicz, H. and Kisza, J. 1976. Studies on the lysozyme content of human and cow milk. Zesz. Nauk. Akad. Roln.-Tech. Olsztynie, Technol. Zywn. **8**:105–112.
80. Grinde, B. 1989. A lysozyme isolated from rainbow trout acts on mastitis pathogens. FEMS Micro. Letters **60**:179–182.
81. Redhead, K., Hill, T. and Mulloy, B. 1990. Antimicrobial effect of human milk on *Bordetella pertussis*. FEMS Micro. Letters **70**:179–182.
82. Carlsson, A., Bjorck, L. and Persson, K. 1989. Lactoferrin and lysozyme in milk during acute mastitis and their inhibitory effect in Delvotest P. J. Dairy Sci. **72**:3166–3175.
83. Hennart, P. F., Brasseur, D. J., Delogne-Desnoeck, J. B., Dramaix, M. M. and Robyn, C. E. 1991. Lysozyme, lactoferrin, and secretory immunoglobulin A content in breast milk: Influence of duration of lactation, nutritional status, prolactin status, and parity of mother. Am. J. Clin. Nutr. **53**:32–39.
84. Shahani, K. M., Chandan, R. C., Kelly, P. L. and MacQuiddy, E. L. 1962. Determination of lysozyme in milk and factors affecting its concentration and properties. Proc. 16th Intern. Dairy Congr. **8**:285–293.
85. Yoshida, A., Takagaki, Y. and Nishimune, T. 1991. Enzyme immunoassay for hen egg white lysozyme used as a food additive. J. Assoc. Off. Anal. Chem. **74**:502–505.
86. Osserman, E. F., Klockars, M., Halper, J. and Fischel, R. E. 1973. Effects of lysozyme on normal and transformed cells. Nature **243**:331.
87. Green, M. L. and Marshall, R. J. 1977. The acceleration by cationic materials of the coagulation of casein micelles by rennet. J. Dairy Res. **44**:521–531.
88. Pearce, K. N. 1976. Moving boundary electrophoresis of native and rennet-treated casein micelles. J. Dairy Res. **43**:27–36.
89. Marshall, R. J. and Green, M. L. 1980. The effect of the chemical structure of additives on the coagulation of casein micelle suspensions by rennet. J. Dairy Res. **47**:359–369.
90. DiGregorio, F. and Sisto, R. 1981. Milk coagulation by cationic polypeptides. J. Dairy Res. **48**:267–271.
91. Giangiacomo, R., Nigro, F., Messina, G. and Cattaneo, T. M. P. 1992. Lysozyme: just an additive or a technological aid as well? Food Additives and Contaminants **9**:427–433.
92. Waugh, D. F. 1971. Formation and structure of casein micelles, p. 3–85. *In*: Milk Proteins Chemistry and Molecular Biology, McKenzie, H. A. (Ed.). Academic Press, New York.
93. Schmidt, D. G. 1982. Association of caseins and casein micelle structure, p. 61–82, Vol. 1. *In*: Developments in Dairy Chemistry, Fox, P. F. (Ed.). Applied Sci Pub., New York.
94. Fox, P. F. 1982. Heat-induced coagulation of milk, p. 189–223, Vol. 1. *In*: Developments in Dairy Chemistry, Fox, P. F. (Ed.) Applied Sci. Pub., New York.
95. Jimenez-Flores, R. and Richardson, T. 1985. Genetic engineering of the caseins to modify the behavior of milk during processing: a review. J. Dairy Sci. **71**:2640–2654.
96. Clark, A. J. 1992. Prospects for the genetic engineering of milk. J. Cell Biochem. **49**:121–127.
97. Yom, H. G. and Bremel, R. D. 1993. Genetic engineering of milk composition: modification of milk components in lactating transgenic animals. Am. J. Clin. Nutr. Suppl. **8**:2995–3065.
98. Schaar, J. 1984. Effect of κ-casein genetic variants and lactation number on the renneting properties of individual milks. J. Dairy Res. **51**:97–406.
99. Marziali, A. S. and Ng-Kwan-Hang, K. F. 1986. Effect of milk composition and genetic polymorphism on coagulating properties of milk. J. Dairy Sci. **69**:1793–1798.
100. Conneely, O. M. and Headon, D. R. 1993. Lactoferrin, lactoperoxidase and lysozyme: nature's protective proteins, p. 123–131. *In*: Biotechnology in the Feed Industry, Proceedings of Alltech's Ninth Annual Symposium, Lyons, T. P. (Ed.). Alltech Tech. Pub., Nicholasville, KY.
101. Arnold, R. R., Cole, M. F. and McGhee, J. R. 1977. A bacteriocidal effect for human lactoferrin. Science **197**:263–265.
102. Kuizinga, A., vanHaeringen, N. J. and Kijlstra, A. 1987. Inhibition of hydroxyl radical formation by human tears. Invest. Ophthalmol. Vis. Sci. **28**:305–313.
103. Masson, P. L. and Heremans, J. F. 1971. Lactoferrin in milk from different species. Comp. Biochem. Physiol. **39B**:143–147.
104. Ellison, R. T. and Giehl, T. J. 1991. Killing gram-negative bacteria by lactoferrin and lysozyme. J. Clin. Invest. **88**:1080–1091.
105. Stuart, J., Norrell, S. and Harrington, J. P. 1984. Kinetic effect of human lactoferrin on the growth of *Escherichia coli* 0111. Int. J. Biochem. **16**:1043–1048.
106. Stephens, S., Dolby, J. M., Montreiul, J. and Spik, G. 1980. Differences in inhibition of the growth of commensal and enteropathogenic strains of *Escherichia coli* by lactoferrin and secretory immunoglobulinA isolated form human milk. Immunology. **41**:597–603.
107. Schulz-Lell, G., Dorner, K., Oldigs, H.-D., Sievers, E. and Scaule, E. 1991. Iron availability from an infant formula supplemented with bovine lactoferrin. Acta. Pediat. Scand. **80**:155–158.
108. Hekman, A. M. 1971. Association of lactoferrin with other proteins, as demonstrated by changes in electrophoretic mobility. Biochim. Biophys. Acta. **251**:380.
109. Maga, E. A., Anderson, G. B. and Murray, J. D. 1995. The effect of mammary gland expression of human lysozyme on the properties of milk in transgenic mice. J. Dairy Sci. *In press.*
110. Gutierrez, A., Maga, E. A., Meade, H. M., Shoemaker, C. F., Medrano, J. F., Anderson, G. B. and Murray, J. D. 1995. Alteration of physical characteristics of milk from bovine kappa-casein transgenic mice. J. Dairy Sci. *Submitted.*